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14. ABSTRACT

Project Summary: The long-term objective of this effort is to develop a generic gene shuff-ling-based technology to rapidly screen libraries of 1010 proteins/peptides encoded by DNA libraries, for identifying biomolecules that can intercept both existing and emerging organo-phosphate-based chemical warfare nerve agents (CWNA). All 5th year milestones have been met: (a) Determine the protective efficacy of 3 PON1 variants in a guinea pig model against GD, GF and GA as compared to untreated animals. Success Criteria: 48hr survival of greater than 90% of the animals tested with 3 nerve agents versus untreated animals. (b) Develop a revised mathematical model to estimate an efficacious dose of a PON1 variant in humans. Success Criteria: Validation of the model against existing guinea pig in vivo efficacy data. (c) Develop at least one PON1 variant with enhanced in vitro and in vivo V-agent activity. Success Criteria: In vitro: a >50 fold increase in hydrolysis of the P- isomer relative to hydrolysis of P (-) VX by 4E9. In vivo: Protection against a 2xLD50 exposure to a V agent in a guinea pig model, as mediated by a 10 mg/kg or lower dose of enzyme administered as a pretreatment. When successful, candidate variants will be used to establish a model to determine in vivo post-exposure efficacy. Relevance: This technology is envisaged to provide rapid discovery of pretreatment and post challenge therapeutic drugs against existing & emerging CWNA threats and will shorten the time from emergence of a threat to identification of potential counter-measures to a few days or weeks.

15. SUBJECT TERMS
Directed evolution, X-ray crystallography, organophosphates & bioscavangers

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Introduction

Our objective is to generate by directed evolution combined with molecular design, the next generation of prophylactic antidotes against chemical warfare nerve agents. These will be catalytic bioscavengers possessing favorable biopharmaceutical properties and capable of hydrolyzing both G- and V-type nerve agents at rates that will qualify them as drug candidates to be used as prophylactic and hopefully also as post-treatment countermeasures against nerve agents intoxication. .

The full protection of animals against multiple LD_{50} doses of nerve agents by pretreatment with stoichiometric scavengers such as acetyl- and butyrylcholinesterase (AChE & BChE,) requires, for example, enhancement of human blood levels of BChE by hundreds of milligrams of exogenously administered human BChE. Thus, the development of catalytic bioscavengers that will permit reasonable protection against G- and V-type chemical warfare intoxication by pretreatment with a substantially lower dose regimen of protein became a mission of high priority. Mammalian paraoxonases (PON1)) became the choice for such endeavor. Yet, for PON1 wt enzymes to be considered as realistic antidotes, the catalytic proficiency requires substantial enhancement of k_{cat}/K_m when reacting with the toxic component of the racemic mixtures of G and V nerve agents.

Over the last 5 years we have demonstrated the potential of directed evolution, combining random and designed mutations based on 3D structures, to generate mutants of a recombinant mammalian PON1 (rePON1) with catalytic proficiency well above the wild type proficiency (Aharoni et al. 2004; Harel et al. 2004; Gupta et al. 2011). We have evolved mutants that hydrolyze more than 10^5-fold faster the toxic isomers of the nerve agents surrogate coumarin-based compounds, when compared to rePON1 and human PON1. We also developed a safe *in situ* protocol for generating the corresponding fluoridates (*i.e.*, the threat G agents), and showed that k_{cat}/K_m values for these variants are as high as $1\text{-}2\times10^7$ $M^{-1}min^{-1}$, a value that approaches the theoretical estimated minimal requirement for efficient prophylactic protection at reasonable doses (~50 mg/70 kg) without the need for post-exposure therapy. The 3rd annual report describes the achievements of the 3rd year highlighted by identification of at least nine potential drug candidates that were over expressed in *E. coli*, purified and transferred to USAMRICD for testing with authentic nerve agents.

Body

I. Specific Aims

1. The development of high-throughput-assays for OP hydrolase variants exhibiting high specificity factors and turnover.

2. Provision of proof-of-concept for the proposed core technology employing directed evolution of new recombinant PON and AChE variants.

3. Isolation of interceptors for G- and V-type nerve agents, and expression in soluble form.

4. Design, generation and selection of 2nd generation libraries for V- and G-type agents.

5. Large-scale production of selected enzyme candidates, and their kinetic, structural and pharmacological evaluation

6. Establishment of "off-the-shelf" libraries for rapid identification of antidotes against emerging future threats

II. Significance to the goals of Counter ACT

The proposed approach opens new opportunities for rapid identification, characterization and implementation of novel countermeasures against CW agents. It will significantly decrease the time interval between the appearance of a new threat and discovery of potential antidotes to counteract it. The major benefits will be one or more products capable of efficient catalytic hydrolysis of G- and V-type nerve agents, as well as gene libraries derived from existing enzymes that can be used "off-the-shelf" to isolate new protein variants for almost any nerve agent or toxic industrial chemical serving as a target for the screen.

III. Year 05 Milestones

In general all 5th year milestones were met, and well beyond.

05 Milestone #2: Determine the protective efficacy (with appropriate statistical significance) of three PON1 variants (identified in Year 4 or during the first 6 months of Year 5) in a guinea pig model against GD, GF and GA as compared to untreated animals. **Success Criteria**: 48 h survival of greater than 90% of the animals tested with 3 nerve agents versus untreated animals.

05 Milestone #3: Develop a revised mathematical model to estimate an efficacious dose of a PON1 variant in humans. **Success Criteria**: Validation of the model against existing guinea pig *in vivo* efficacy data.

05 Milestone #4: Develop at least one PON1 variant with enhanced *in vitro* and *in vivo* V-agent activity. **Success Criteria:** *In vitro*: a >50 fold increase in hydrolysis of the P-isomer relative to hydrolysis of P(-) VX by variant 4E9. *In vivo*: Protection against a 2xLD50 exposure to a V agent in a guinea pig model, as mediated by a 10 mg/kg or lower dose of enzyme administered as a pretreatment. When successful, candidate variants will be used to establish a model to determine *in vivo* post-exposure efficacy

In our previous annual reports (for Years 1-4) we described an enhanced evolution strategy, combining random and designed mutations, to yield several mammalian rePON1 mutants with catalytic proficiency approaching the value that qualifies a catalytic bioscavenger as a candidate drug for pre-treatment against OP intoxication ($k_{cat}/K_m \sim 5x10^7\ M^{-1}min^{-1}$). Earlier, we increased the sensitivity of our screen for clones capable of hydrolyzing the toxic isomers of CMP-coumarin and of its fluoridate analog, GF (see report for Year 4), using the AChE inhibition protocol on whole-cell lysates, *i.e.*, the Interception Screening Approach (ISA). The successful implementation of the ISA protocol prompted the generation and screening of two new libraries, R3 and R4 (Table 1), the design of which was based, in part, on the crystal structure of the penta-mutant 2D8 (L69G, H115W, H134R, F222S, T332S). The round 3 (R3) library was constructed by shuffling the 7 best clones of the previous round with oligonucleotides encoding for specific mutations in either the protein's active site (ancestral or second-shell mutations) or in a surface loop, positions 69-82, adjacent to the active site. The round 4 (R4) library was constructed by shuffling the 18 most improved variants from round 3. Gene shuffling of improved variants enabled us to combine beneficial active site and second-shell mutations with mutations in the surface loop-region. Clones from the R3 and R4 libraries were screened using *in situ*-generated GB and GD. Table 1 shows that the most improved R3 variant was ~1.5-fold more active than the best R2 variant towards the 2 toxic isomers of GD, and 3-fold more active towards GF and GB. The R4 library evolved even more effective rePON1 variants, of which the most active, VIID2, displayed k_{cat}/K_m values of $7\text{-}9x10^7\ M^{-1}min^{-1}$ for the detoxification. Despite the remarkable enhancement in rePON1 proficiency towards GD and GF (9000- and 540-fold respectively), relative to the wt G3C9 variant, the improvement in GB hydrolysis was only 20-40-fold, suggesting that the relatively small O-isoPr moiety did not produce the tight accommodation required for effective formation of a productive complex within the catalytic

site. Further rounds of evolution will be required to further increase the k_{cat}/K_m for GB hydrolysis so as to produce an effective 'general ist' rePON1 scavenger. It is noteworthy that VIID2 hydrolyzes both toxic isomers of GD rapidly and at equal rates ($9.2x10^7$ $M^{-1}min^{-1}$).

The most active R4 variants (Round 4 librar y - Tab le 1) share several mutations: L69V, H115A, H134R, F222M, I291L and T332S (see Tabl e 2). The least active R4 variant, VI-E4 differs principally in bearing a Y71C m utation on the flexible loop, as well as the D136H mutation (Table 2).

The evolution of V-type hydrol yzing variants has lagged behi nd the development of G-type hydrolyzing variants primarily due to the lack of suitable V-agent su rrogates. Variants that failed to hydrolyze the O-diethylphosphoryl an alog of V-type nerve agents, am iton $(C_2H_5O)_2P(O)SCH_2CH_2N(Et)_2$, Fig. 1) were subsequently found to exhibit low, yet detectable, activity on the O-alkyl methylphosphonothiolates (*e.g.*, VM, VX, RVX). Vari ants belonging to earlier generations (*e.g.*, wt-G3C9, 3B3, 1A4, 0C9, 2D8) , hydrolyzed VM at 2-5 m U/mg compared to 40 m U/mg displayed by bacteria l wt PTE. Am iton is hydrolyzed by PTE (50 mU/mg) but not by the rePON1 variants m entioned above. Thus, VM, rather than am iton, can serve as a suitable VX analog for identification of rePON1 variants capable of hydrolyzing P-S - alkyl containing nerve agents. In order to develop an effi cient screening procedure for identification of varian ts capable of efficiently degrading V agents, we again utilized the ISA approach with hAChE added to the lysate s. For this purpose, we developed an *in situ* non-hazardous synthetic pro cedure for the generatio n of two pairs of m ethylphosphonothiolates that differ in the size of the O-alkyl moiety, namely VX and its hom olog VM, and RVX and its homolog RVX-isoPr (Fig. 1). Our first (Round 1) VX library was m ade by screening lysates of the R4 G agent lib rary (Table 2) with VX, u tilizing the I SA protocol based on inhibition of hAChE. We isolated and characterized 3 im proved variants from that round, which displayed >200-fold enhanced hydrolysis of VX relative to wt-rePON1 (Table 3). W e then used VX to assay the catalytic efficiency of variants isolated and purified from all previous rounds of G-agent evolution. Although variants from earlier rounds of G-type evolution were less active on VX, they had dif ferent mutational compositions. We therefore combined the 4 varian ts from earlier rounds with the highest activity towards VX with the 3 most active R4 variants referred to above, which were denoted VX-R1, and shuffled th em together to generate a Round 2 library of VX mutants (VX-R2 library). This library was screened again against VX and RVX, and it was

possible to isolate and characterize 4 variants that showed significantly improved VX hydrolysis. The best VX-R2 variant was S-2-C8 (Appendices #4 & 5). In conclusion, efforts in this milestone have resulted, so far, in at least one variant capable of hydrolyzing the toxic isomer of VX >5000-, >130- and >25-fold better than wt-G3C9, 4E9 and the VX-G1 variants, respectively (Table 3). Thus, S-2-C8 hydrolyzed the toxic isomer of VX at $1.0x10^4$ M^{-1} min^{-1}, a value similar to that for a bacterial PTE that we had used to verify the interception protocol with V agents (Table 3). To gain further insight into the stereo-preference of S-2-C8 when reacting with the racemic VX, release of the leaving group, HS CH2CH2N(isoPr)2,was monitored by use of Ellman's reagent, DTNB. Fig. 2 clearly shows that the two optical isomers of VX are hydrolyzed equally well by S-2-C8. A similar kinetic time course, albeit slower, was observed with VM (not shown). RVX and RVX-isPr were found, however, to be poor substrates, suggesting that although the binding pocket in this variant accommodates the O-ethyl moiety well, it needs to be enlarged to permit enhanced hydrolysis of the O-isobutyl-containing V agents.

In summary, the year 5 data provide improved catalytic scavengers with proficiencies that exceed the initial goal (*i.e.*, $k_{cat}/K_M > 5x10^7$ $M^{-1}min^{-1}$) and illustrate the potential to systematically improve the proficiency of rePON1, and to obtain a PON1 variant that will serve as a universal G-agent catalyst. Library VX-R2 opens the way to evolution of proficient variants capable of detoxifying V agents

Additional progress in year 05:

Ex vivo protocol: In order to evaluate the stability and proficiency of rePON1 variants in human blood we developed an *ex-vivo* protocol to screen the potential antidotal capacity of rePON1 variants via their protection of endogenous acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in human whole blood obtained from a local blood bank. This protocol permitted us to address the relationship between blood rePON1 concentrations, together with their kinetic parameters, and the level of protection conferred on the ChEs in human blood, following a challenge with GF (Fig. 3). The GF was generated *in situ* at a non-hazardous concentration. In addition, long-term incubation of the tested rePON1 variants with whole blood, prior to spiking with GF, provided information concerning the effect of their association with

blood components on their stability. For example, *ex-vivo* treatment of human whole blood with 0.45 μM VIID2 (an R4 variant) resulted in retention of 42% of the total ChE activity following spiking with 0.08 μM GF, compared to only 5% in unprotected blood. These experimental data are in good agreement with the % residual activities calculated on the basis of the rate constants for inhibition of human AChE and BChE by GF, the concentration of the rePON1 variant, and its k_{cat}/K_m value. The validation protocol developed provides a rapid and reliable *ex-vivo* screening tool for selection of rePON1 bioscavenger candidates suitable for use in the protection of humans against OP intoxication. The results also provide input for a revised mathematical model for estimating the efficacious dose required in humans of a given rePON1 variant.

Key Research Accomplishments

- Five mutants capable of hydrolyzing the toxic isomers of GD and GF with k_{cat}/K_M values of 2-9x10^7 $M^{-1}min^{-1}$: 1-I-F11, 1-IV-H9, IIG1, VH3, VIID2
- New libraries, R3 and R4, of variants that effectively hydrolyze G agents.
- New libraries, VX-R1 and VX-R2, of variants capable of hydrolyzing VX and VM
- A protocol for a controlled, safe and non-hazardous procedure to generate VM, VX, RVX and RVX-isoPr
- A protocol for *ex-vivo* estimation of k_{cat}/K_M values of rePON1 variants in human blood

Reportable Outcomes

Manuscripts:

1. Ashani, Y., Gupta, R.D., Goldsmith, M., Silman, I., Sussman, J.L., Tawfik, D.S. & Leader, H. (2010). "Stereo-specific synthesis of analogs of nerve agents and their utilization for selection and characterization of Paraoxonase (PON1) catalytic scavengers" *Chem Biol Interact* **187**, 362-369 (attached).
2. Dvir, H., Silman, I., Harel, M., Rosenberry, T.L. & Sussman, J.L. (2010). "Acetylcholinesterase: From 3D structure to function " *Chem Biol Interact*, **187**, 10-22 (attached).
3. Gupta, RD., Goldsmith , M., Ashani ,Y, Simo Y, Mullokandov G, Bar H, Ben-David M, Leader H, Margalit R, Silman I, Sussman JL, Tawfik DS (2011) "Directed evolution of hydrolases for prevention of G-type nerve agent intoxication" *Nat Chem Biol* **7**, 120-125 (attached).

Conclusions

The model OP compounds and the *in situ* generation of G agents together with the screening approach that we have developed and the libraries that have been generated, establish our methodologies for future screening and identification of promising PON variant candidates. Several promising candidates have been transferred to ICD to be tested with threat agents.

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Gupta, R.D., Goldsmith, M., Ashani, Y., Simo, Y., Mullokandov, G., Bar, H., Ben-David, M., Leader, H., Margalit, R., Silman, I., Sussman, J.L., and Tawfik, D.S. 2011. Directed evolution of hydrolases for prevention of G-type nerve agent intoxication. *Nat. Chem. Biol.* **7:** 120-125.

Harel, M., Aharoni, A., Gaidukov, L., Brumshtein, B., Khersonsky, O., Meged, R., Dvir, H., Ravelli, R.B., McCarthy, A., Toker, L., Silman, I., Sussman, J.L., and Tawfik, D.S. 2004. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat. Struct. Mol. Biol.* **11:** 412-419.

Tables

Table 1. Best variants obtained in each round of evolution based on activity towards G-type nerve agents

Variant	round	GD[a,b] k_{cat}/K_M x10^7 [$M^{-1}min^{-1}$]		GF[a] k_{cat}/K_M x10^7 [$M^{-1}min^{-1}$]	GB[a] k_{cat}/K_M x10^7 [$M^{-1}min^{-1}$]
		Fast	Slow		
rePON1-G3C9	-	0.0043	0.001	0.013	0.008
8C8	0	0.0028	0.0014	0.021	0.003
0C9	0	0.34	0.026	1.11	0.032
1A4	0	0.41	0.033	1.13	0.021
2D8	0	0.411	0.015	1.43	0.023
4E9	0	0.74	0.056	1.68	0.03
Fold improvement R0 [c]		*172*	*56*	*129*	*4*
5H8	1	0.57	0.064	0.244	0.01
PG11	1	1.4	0.32	0.212	0.02
Fold improvement R1 [c]		*326*	*320*	*16*	*3*
VI-D2	2	1.4	0.33	0.28	0.02
MG2-I-A4	2	1.95	1.3	1.28	0.13
IV-D11	2	2.5	0.73	1.06	0.13
II-A1	2	2.5	0.73	0.59	0.07
V-B3	2	2.6	0.68	1.2	0.05
VII-D11	2	2.9	2.9	1.07	0.12
Fold improvement R2 [c]		*674*	*2900*	*82*	*15*
2-II-D12	3	0.27	0.27	0.48	0.18
1-I-D10	3	0.87	0.87	1.36	0.31
I-IV-H9	3	4.2	4.2	2.63	0.31
1-I-F11	3	4.4	4.4	1.52	0.39
Fold improvement R3 [c]		*1023*	*4400*	*117*	*49*
IIG1	4	5.5	5.5	3.6	0.32
VH3	4	8.4	2.6	2.72	0.3
VIID2	4	9.2	9.2	7.00	0.17
Fold improvement R4 [c]		*2139*	*9200*	*540*	*21*

a. *in situ* generated G-agent. Values relate to action on the toxic *S*p isomer.
b. Fast and slow hydrolysis of the two equally toxic isomers of GD
c. Improvement for best variant in the round relative to rePON1-G3C9

Table 2 Summary of mutations found in improved clones from the Round 4 library

Residue #	rePON1	VII-D2	II-G1	V-H3	I-B10	II-E11	VI-A2	VI-E4
55	L	I	I	L	V	V	L	I
69	L	V	V	V	V	V	L	V
70	K	K	K	K	K	K	N	K
71	Y	Y	Y	Y	Y	Y	Y	C
115	H	A	A	A	A	A	A	A
134	H	R	R	R	R	R	R	R
136	D	D	Q	D	D	D	D	H
197	H	R	H	H	H	H	H	H
222	F	M	M	M	M	M	M	M
291	I	L	L	L	F	L	L	L
332	T	S	S	S	S	S	S	S

Table 3.

Catalytic Activity (k_{cat}/K_m, $M^{-1}min^{-1}$) of PON1 Variants hydrolyzing the toxic isomers of V-type agents (inhibition of hAChE)

Variant	Round	VX	RVX
Wt-G3C9		<2	<2
8C8	VX-G0	93	
4E9	**VX-G0**	**80**	
VIIH3	VX-R1	413	327
VA4	VX-R1	487	249
IA12	VX-R1	288	114
S-2-C8	**VX-R2**	**10,600**	<200

NOTE: Bacterial PTE hydrolyzed VX at 8,500 $M^{-1}min^{-1}$

Figures

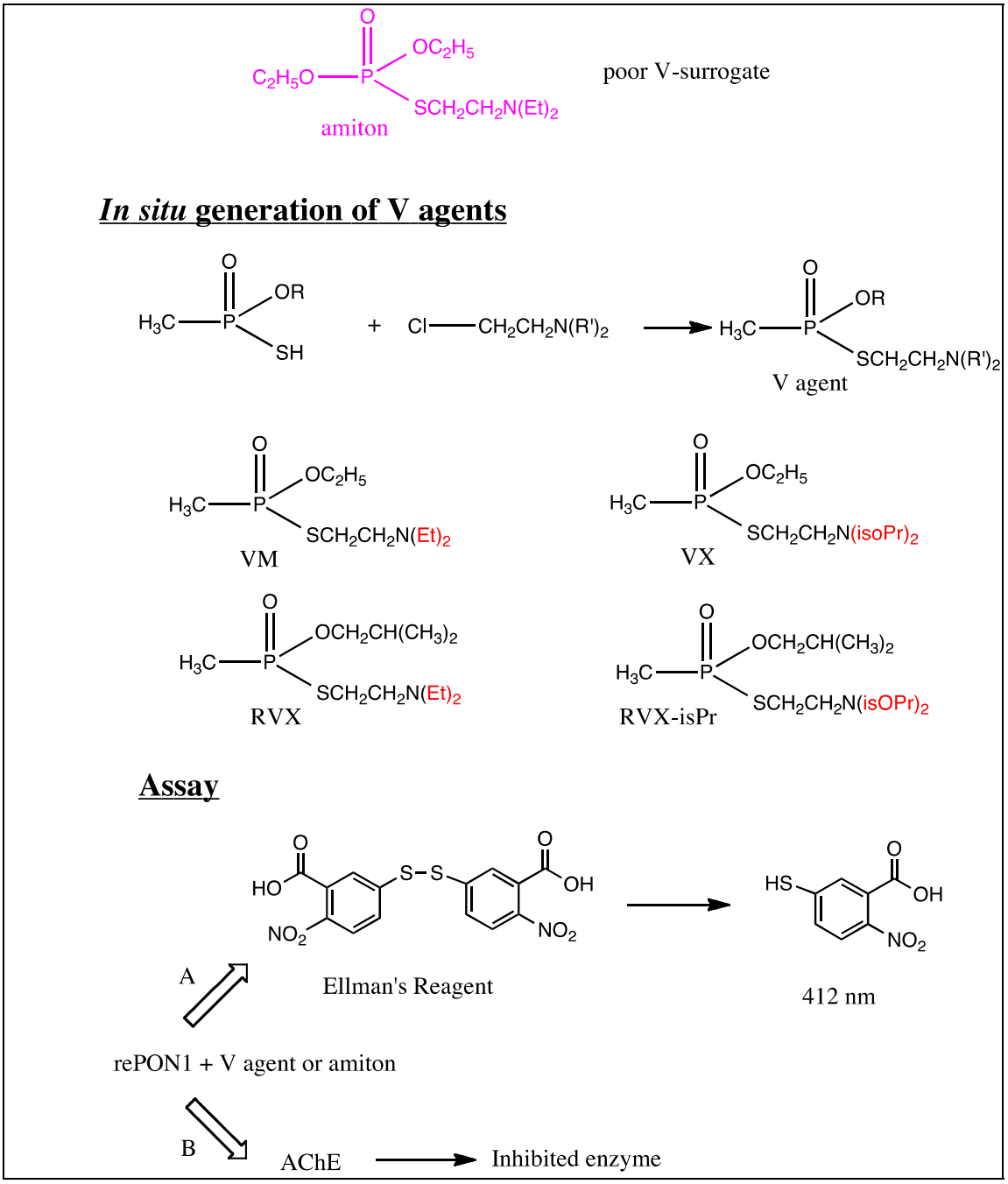

Figure 1. Synthesis and structures of V-type nerve agents

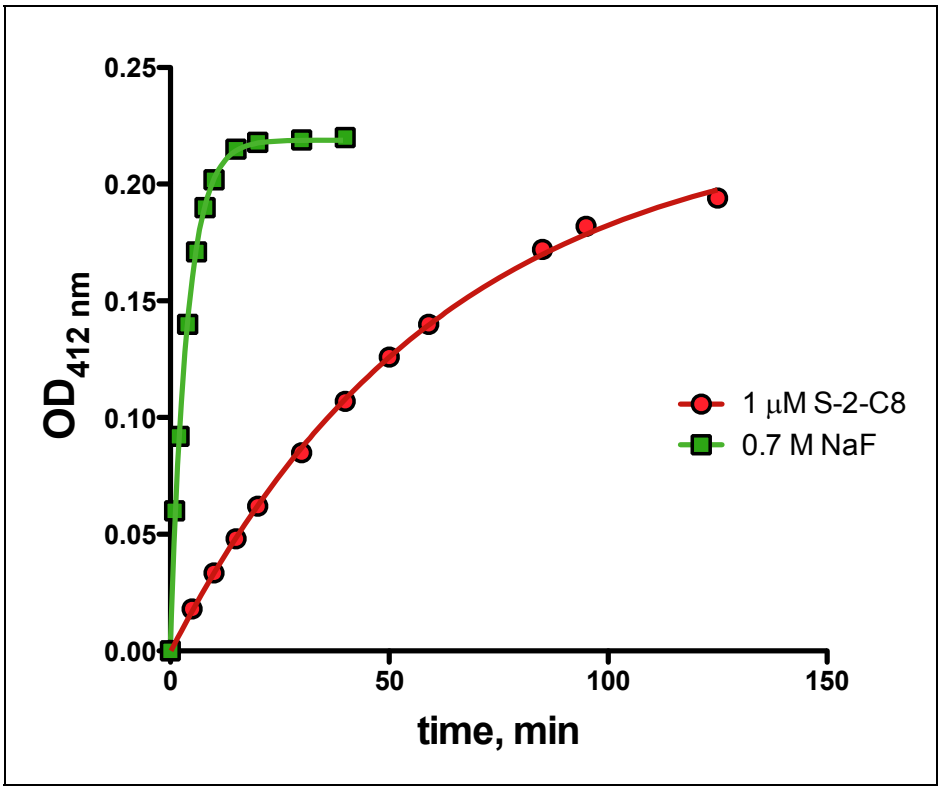

Figure 2. S-2-C8-induced hydrolysis of 1.7x10 $^{-5}$ M VX using DTNB to capture the leavin g group

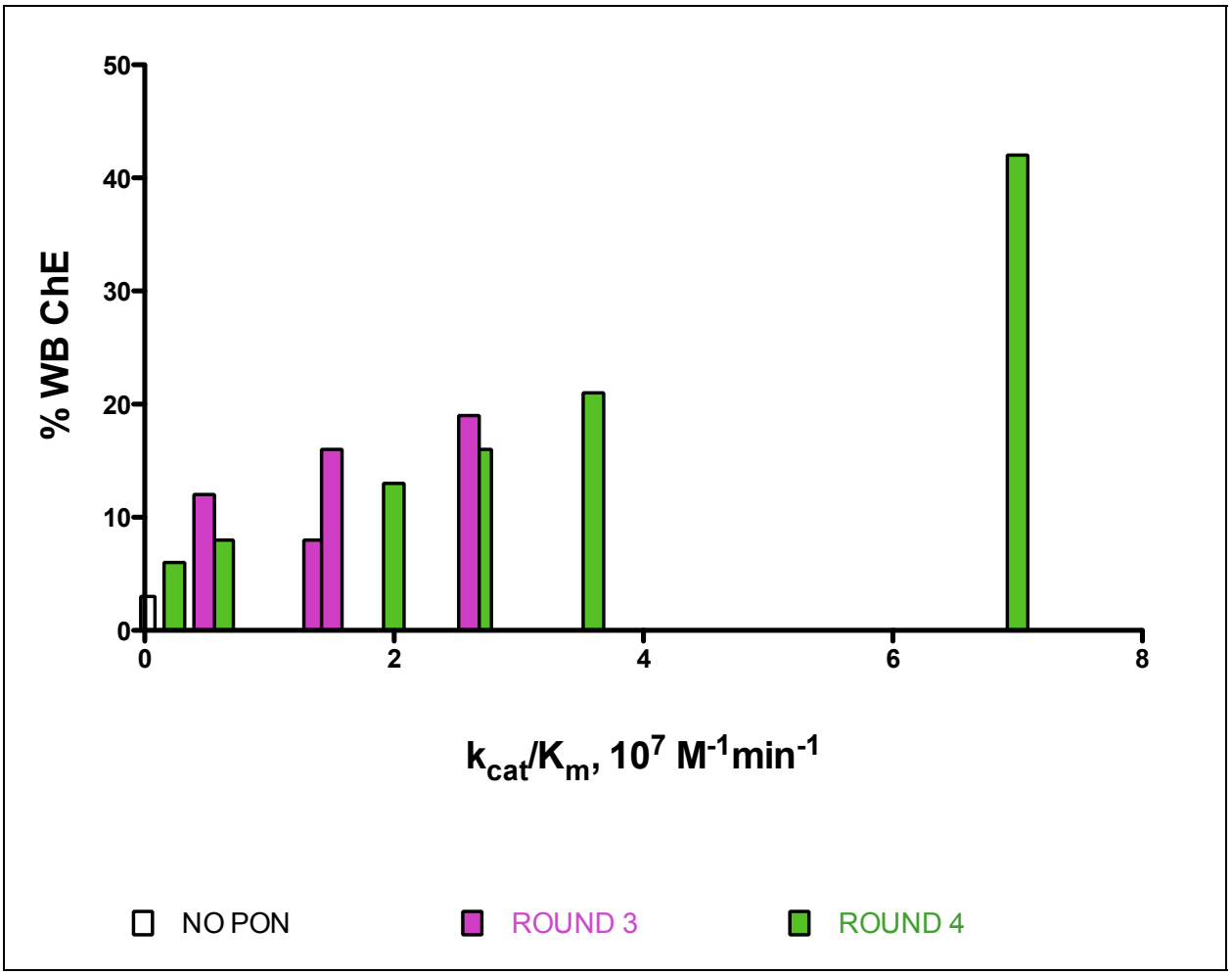

Figure 3. Correlation between protec tion of whole blood (W B) cholinesterase activity (AChE+BChE) and the k_{cat}/K_m values of rePON1 variants from Rounds 3 and Round 4 (at equimolar concentrations) determined in buffer solution (GF concentration 0.080 μM)